Genotyping of Non-MHC Loci in Nonhuman Primates

Prepared by: David O'Connor, Professor, and Julie Karl, Senior Research Specialist

May 1, 2024

UW-Madison WNPRC 555 Science Drive Madison, WI 53711 **T** (608) 890-0847 **F** (608) 265-8084 uwgenomicservices@primate.wisc.edu

Genotyping of Non-MHC Loci

Introduction

We recognize that many investigators utilizing nonhuman primates to study cellular immunity are interested in loci beyond the MHC. We currently offer *TRIM5* genotyping using a newly developed MiSeq short amplicon sequence-based assay to detect *TRIM5*^{Q}, *TRIM5*^{TFP}, and *TRIM5*^{CypA} alleles. We are also developing PacBio Sequel protocols for full-length sequencing of other immune loci, such as Fc gamma receptor (FcγR) and killer immunoglobulin-like receptor (KIR) genotyping. Though FcγR and KIR assays are still under development for most nonhuman primate populations, we are willing to work with investigators interested in prototyping these assays as "early access" clients.

TRIM5 Genotyping

Tripartite motif protein 5 alpha (TRIM5α) in primates such as humans, apes, macaques, and new world monkeys, as well as other mammals, is an important restriction factor to potentially suppress retroviral replication. The rhesus macaque *TRIM5* gene is highly polymorphic, and it has been shown that specific *TRIM5* alleles can affect acquisition and replication of simian immunodeficiency virus (SIV) replication. While there are multiple allelic variants, they can be grouped into three functional classes based on their C-terminal B30.2/SPRY domain: *TRIM5*^{Q}, *TRIM5*^{TFP}, and *TRIM5*^{CypA}.

Starting material for genotyping

For *TRIM5* genotyping, we require one of the following:

- 20 µl of genomic DNA at a concentration of ≥60 ng/µl
- 2 ml of fresh or frozen whole blood in EDTA tubes
- 5 x 10^6 cryopreserved PBMC

Genomic DNA samples may be shipped overnight at room temperature. Blood samples can be shipped either fresh or frozen; fresh blood samples should be shipped overnight with cold packs, and frozen blood samples should be shipped overnight on dry ice. Cell samples should be shipped overnight on dry ice. Genomic DNA isolations will be performed on whole blood or cells using a Promega Maxwell instrument.

PCR amplification

Two short amplicons are generated for *TRIM5* sequence-based genotyping. The first amplicon covers the *TRIM5*^{Q} and *TRIM5*^{TFP} polymorphism. The second amplicon spans an altered splice site at the 3' end of intron 6 that is responsible for alternative splicing to generate the *TRIM5*^{CypA} variant. These amplicons are included with our standard Fluidigm Access Array primer panel for MiSeq MHC genotyping. MiSeq sequencing adapters and sample-specific barcodes are added to each amplicon, and pools of amplicons are harvested from the microfluidics chip. Pooled amplicons are SPRI-bead purified, quantitated, and normalized.

Sequencing: Illumina MiSeq

We generally pool barcode-tagged *TRIM5* amplicons with MHC class I and class II amplicons into a single pool for MiSeq sequencing. Cluster formation is performed on-instrument. Additional details about the MiSeq instrument system and sequencing workflow can be found on the Illumina website (http://www.illumina.com/systems/miseq.ilmn).

Analysis of PCR products

The amplicon sequences are analyzed for Q versus TFP amino acids for the *TRIM5*^{Q} and *TRIM5*^{TFP} amplicon, and for presence of the alternative splice site for the *TRIM5*^{CypA} amplicon. A report like the one below is generated to summarize the sequence analysis.

Animal ID	*TRIM5* Allele 1	*TRIM5* Allele 2
XY001	TFP	TFP
XY002	TFP	TFP
XY003	TFP	CypA
XY004	TFP	CypA
XY005	CypA	CypA
XY006	Q	TFP
XY007	Q	Q
XY008	TFP	CypA

Costs for *TRIM5* Genotyping

Genotyping costs per animal are shown in the tables below. **These rates are effective from May 1, 2024 through April 30, 2025.** Rates are subject to change annually. Direct cost recovery is used to set the base rate for University of Wisconsin clients (Tier 1). For clients outside of the UW, we must also recover indirect costs (facilities & administrative overhead, etc.). The current rates for these indirects are 38.5% for other US federal and non-profit entities (Tier 2), 55.5% for foreign universities and non-profits (such as foreign NIH equivalents, e.g. the UK's NHS) and US for-profit entities using federal funds, and 72.5% for for-profit entities (Tier 3). A more detailed breakdown of costs is available upon request.

TRIM5 genotyping alone

	Whole Blood	gDNA
Reagents Total/Animal	$35	$21
Labor Total/Animal	$58	$49
Total Costs	**$93**	**$70**
Tier 1 - UW-Madison	**$93.00**	**$70.00**
Tier 2 - Federal & Non-Profit	**$128.81**	**$96.95**
Tier 2a - Specialty Non-Profit	**$144.62**	**$108.85**
Tier 3 - For-Profit	**$160.43**	**$120.75**

Standard turnaround time for all *TRIM5* genotyping assays is 8-12 weeks from receipt of samples. These assays can also be expedited at a 50% premium on top of the Tier 1 rates (plus any applicable overhead; expedited *TRIM5* genotyping from whole blood, for instance, would be available for $139.50 to Tier 1 clients, $193.21 for Tier 2 clients, etc.). The extra charge is to cover the rush sample processing, and reduces turnaround to 4-6 weeks.

Alternatively, *TRIM5* genotyping can be performed at the same time as MiSeq short-amplicon MHC genotyping for a set of samples for a nominal fee.

TRIM5 genotyping concurrent with MiSeq MHC genotyping (Mauritian cynomolgus macaques)

	Whole Blood	gDNA
Reagents Total/Animal	$52	$39
Labor Total/Animal	$82	$73
Total Costs	**$134**	**$112**
Tier 1 - UW-Madison	**$134.00**	**$112.00**
Tier 2 - Federal & Non-Profit	**$185.59**	**$155.12**
Tier 2a - Specialty Non-Profit	**$208.37**	**$174.16**
Tier 3 - For-Profit	**$231.15**	**$193.20**

TRIM5 genotyping concurrent with MiSeq MHC genotyping (Indian rhesus macaques and other nonhuman primate species)

	Whole Blood	gDNA
Reagents Total/Animal	$52	$39
Labor Total/Animal	$107	$99
Total Costs	**$159**	**$138**
Tier 1 - UW-Madison	**$159.00**	**$138.00**
Tier 2 - Federal & Non-Profit	**$220.22**	**$191.13**
Tier 2a - Specialty Non-Profit	**$247.25**	**$214.59**
Tier 3 - For-Profit	**$274.28**	**$238.05**

Contact Information

For more information or to discuss the specifics of your project, please contact the UW-Madison WNPRC Genomics Services unit at uwgenomicservices@primate.wisc.edu. Before starting any genotyping, we require execution of a service agreement (example appended to the end of this document) and generation of a purchase order. An intake form must also be filled out prior to starting any service. For any additional information, please see our website (https://primate.wisc.edu/research-services/genomics-services/).

Fc Gamma Receptor Genotyping

Fc receptors (FcR) are glycoproteins expressed on immunologically active cells that recognize and bind the Fc portions of immunoglobulins. When immunoglobulin particles like pathogens, autoantigens, and allergens bind to these receptors, immune-competent cells are ultimately activated or inhibited. One family of these receptors, the IgG Fc receptors (FcγR), exhibit multiple allelic polymorphisms and have distinct expression patterns, recognition, and activation profiles of their protein products. There are three classes of FcγR - FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). In humans, a total of eight FcγR genes have been identified (three FcγRI, two FcγRII, and two FcγRIII). Very little is known about the FcγR region of macaques.

We are developing PacBio Sequel protocols for full-length sequencing of FcγR alleles from RNA transcripts. These protocols are similar to our PacBio MHC genotyping protocols, with primers located in relatively conserved regions of the UTRs surrounding the FcγR loci. FcγR PacBio genotyping was piloted in Mauritian cynomolgus macaques, a population with an overall restricted diversity which greatly simplifies data analysis. We are now beginning to establish a database of Indian rhesus macaque FcγR alleles. These protocols are still under development. Investigators that are interested in potentially providing samples for FcγR genotyping method development should contact the UW-Madison Genomics Services unit at uwgenomicservices@primate.wisc.edu

Starting material for genotyping

For FcγR PacBio genotyping, we require one of the following:

- 25 µl of total RNA at a concentration of ≥10 ng/µl
- 20 µl of cDNA synthesized using the Superscript III First-Strand Synthesis System for RT-PCR from Invitrogen
- 2 ml of fresh whole blood in EDTA tubes
- 5 x 10^6 cryopreserved PBMC

cDNA samples may be shipped overnight at room temperature. RNA and cell samples should avoid freeze-thaw cycles and should be shipped overnight on dry ice. Whole blood samples should be shipped overnight with cold packs. These conditions must be followed to minimize degradation of RNA from the starting material during shipping. Total RNA isolations will be performed on whole blood or cells using a Promega Maxwell instrument.

Sample processing overview

We use 50 ng of total RNA as a template for first-strand cDNA synthesis using the Superscript III First-Strand Synthesis System for RT-PCR from Invitrogen. This cDNA is then utilized to generate cDNA-PCR amplicons spanning the entire full- length coding region of FcγR alleles. In our pilot experiments with Mauritian cynomolgus macaques, we detected alleles at four loci: FcγRI, FcγRIIa, FcγRIIb, and FcγRIII. Four locus-specific PCR reactions are thus performed for each sample. PacBio barcode identifier sequence tags are incorporated via the PCR primers, and amplicons are SPRI-bead purified, quantitated, and normalized. We pool the amplicons (all four loci) for up to 32 animals together into a single pool for PacBio SMRT sequencing. PCR products are circularized prior to long-read sequencing on a Sequel instrument. Additional details about the Sequel instrument system and sequencing workflow can be found on the PacBio website (http://www.pacb.com/products-and-services/pacbio-systems/). PacBio sequence reads are binned by barcode tag and filtered to remove insertion/deletion base calling artifacts and short reads. Filtered reads are then processed to cluster identical sequences together. Clusters supported by 3 or more independent reads are manually curated and saved to create a species-specific database of FcγR alleles.

Costs for FcγR Genotyping

Genotyping costs per animal are shown in the tables below. **These rates are effective from May 1, 2024 through April 30, 2025.** Rates are subject to change annually. Direct cost recovery is used to set the base rate for University of Wisconsin clients (Tier 1). For clients outside of the UW, we must also recover indirect costs (facilities & administrative overhead, etc.). The current rates for these indirects are 38.5% for other US federal and non-profit entities (Tier 2), 55.5% for foreign universities and non-profits (such as foreign NIH equivalents, e.g. the UK's NHS) and US for-profit entities using federal funds, and 72.5% for for-profit entities (Tier 3). A more detailed breakdown of costs is available upon request.

There are two rate structures for the FcγR genotyping assay. A full database of FcγR alleles in Mauritian cynomolgus macaques has been completed, due to their overall limited genetic diversity. This simplifies data analysis and reduces analysis costs for that population. For all other nonhuman primate populations, "early access" client samples will be used to build the allele databases, and additional primers may need to be developed for other populations.

	Mauritian Cynomolgus Macaques	Other Nonhuman Primates
Reagents Total/Animal	$92	$92
Labor Total/Animal	$105	$150
Total Costs	**$197**	**$242**
Tier 1 - UW-Madison	**$197.00**	**$242.00**
Tier 2 - Federal & Non-Profit	**$272.85**	**$335.17**
Tier 2a - Specialty Non-Profit	**$306.34**	**$376.31**
Tier 3 - For-Profit	**$339.83**	**$417.45**

Killer Immunoglobulin-Like Receptor Genotyping

Killer immunoglobulin-like receptors (KIRs) are cell surface receptors expressed on Natural Killer (NK) cells and some T lymphocytes. KIRs interact with their ligands to mediate activating or inhibitory signals and are an important component in recognizing self versus foreign cells and priming the adaptive immune system. MHC class I molecules are the primary ligands for KIRs. Specific combinations of MHC and KIR alleles in humans have been associated with susceptibility to autoimmune diseases and protection from infectious diseases like HIV and malaria. Like the macaque MHC region, the KIR gene region contains variable numbers of expressed alleles, and KIR alleles are highly polymorphic. For most nonhuman primate populations, the KIR region is largely uncharacterized.

We are also developing PacBio Sequel protocols for full-length sequencing of KIR alleles from RNA transcripts, with primers located in the UTRs surrounding the KIR loci. KIR PacBio genotyping is being piloted in Mauritian cynomolgus macaques at this time, with Indian rhesus macaques to follow. As with FcγR typing, investigators interested in becoming an "early access" client during KIR genotyping assay development should contact the UW-Madison Genomics Services unit at uwgenomicservices@primate.wisc.edu.

Starting material for genotyping

For KIR PacBio genotyping, we require one of the following:

- 25 µl of total RNA at a concentration of ≥10 ng/µl
- 20 µl of cDNA synthesized using the Superscript III First-Strand Synthesis System for RT-PCR from Invitrogen
- 2 ml of fresh whole blood in EDTA tubes
- 5 x 10^6 cryopreserved PBMC

cDNA samples may be shipped overnight at room temperature. RNA and cell samples should avoid freeze-thaw cycles and should be shipped overnight on dry ice. Whole blood samples should be shipped overnight with cold packs. These conditions must be followed to minimize degradation of RNA from the starting material during shipping. Total RNA isolations will be performed on whole blood or cells using a Promega Maxwell instrument.

Sample processing overview

We use 50 ng of total RNA as a template for first-strand cDNA synthesis using the Superscript III First-Strand Synthesis System for RT-PCR from Invitrogen. This cDNA is then utilized to generate cDNA-PCR amplicons spanning the entire full- length coding region of KIR alleles. In our pilot experiments with Mauritian cynomolgus macaques, we detected KIR1D, KIR3DL, and KIR3DH alleles with a single pair of primers, and KIR2DL alleles with a second primer. Additional primer design may be required for other nonhuman primate species. PacBio barcode identifier sequence tags are incorporated via the PCR primers, and amplicons are SPRI-bead purified, quantitated, and normalized. We pool the amplicons for up to 32 animals together into a single pool for PacBio SMRT sequencing. PCR products are circularized prior to long-read sequencing on a Sequel instrument. Additional details about the Sequel instrument system and sequencing workflow can be found on the PacBio website (http://www.pacb.com/products-and-services/pacbio-systems/). PacBio sequence reads are binned by barcode tag and filtered to remove insertion/deletion base calling artifacts and short reads. Filtered reads are then processed to cluster identical sequences together. Clusters supported by 3 or more independent reads are manually curated and saved to create a species-specific database of KIR alleles.

Costs for KIR Genotyping

Genotyping costs per animal are shown in the tables below. **These rates are effective from May 1, 2024 through April 30, 2025.** Rates are subject to change annually. Direct cost recovery is used to set the base rate for University of Wisconsin clients (Tier 1). For clients outside of the UW, we must also recover indirect costs (facilities & administrative overhead, etc.). The current rates for these indirects are 38.5% for other US federal and non-profit entities (Tier 2), 55.5% for foreign universities and non-profits (such as foreign NIH equivalents, e.g. the UK's NHS) and US for-profit entities using federal funds, and 72.5% for for-profit entities (Tier 3). A more detailed breakdown of costs is available upon request.

There are two rate structures for the KIR genotyping assay. An extensive database of KIR alleles in Mauritian cynomolgus macaques is already available, as described in Prall et al. Immunogenetics 69: 325-339 (2017) and Bimber et al. J Immunol. 181: 6301-8 (2008). "Early access" client samples for all other nonhuman primate populations will again be used to build allele databases and optimize protocols for non-Mauritian animals.

	Mauritian Cynomolgus Macaques	Other Nonhuman Primates
Reagents Total/Animal	$86	$86
Labor Total/Animal	$144	$231
Total Costs	**$230**	**$317**
Tier 1 - UW-Madison	**$230.00**	**$317.00**
Tier 2 - Federal & Non-Profit	**$318.55**	**$439.05**
Tier 2a - Specialty Non-Profit	**$357.65**	**$492.94**
Tier 3 - For-Profit	**$396.75**	**$546.83**